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Replacement of Soybean Meal with Fermented *Jatropha curcas* Kernel Meal in the Diet of *Labeo rohita* Fingerlings: Effect on Hemato-biochemical and Histopathological Parameters

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Abstract

***Jatropha* kernel meal was detoxified through the process of solid-state fermentation using *Aspergillus niger* fungus. A 60-d feeding trial was conducted to evaluate the effects of replacing soybean meal (SBM) with fermented Jatropha kernel meal (FJKM) on the hemato-biochemical and histopathological parameters in *Labeo rohita* fingerlings (average weight 6.05 ± 0.04 g). A total of 180 fish were randomly distributed in 12 experimental tanks in four dietary groups, namely control (SBM-based diet), T1 (10% FJKM replacing 33.3% SBM), T2 (20% FJKM replacing 66.7% SBM), and T3 (30% FJKM replacing 100% SBM). There was no significant difference in the red blood cell, white blood cell, hemoglobin, total protein, albumin, globulin, and respiratory burst activity among the various groups, but significantly higher hematocrit value and albumin/globulin ratio were observed in the T2-fed group compared with the control. No statistical difference was found in serum glucose level of the control and FJKM-fed groups. Histopathological examination of the intestine and liver indicated no adverse effects in the control and FJKM-fed groups. Our findings revealed that FJKM can replace 100% SBM protein in the diets of *L. rohita* fingerlings, without any adverse effects on fish health.**

Soybean meal (SBM) is one of the most researched and commonly used alternative plant protein sources in animal diets (Yue and Zhou 2008) because of its relatively high protein contents, rich amino acid composition, and digestibility (Hertrampf and Piedad-Pascual 2000). The rapid expansion of feed-based aquaculture, especially in India, and increasing cost of fishmeal (FM) have fueled the demand for this high-quality plant protein source. However, the use of SBM has become a limitation because of its increasing cost, competition with human/livestock, and availability. This situation has limited the expansion and profitability of aquaculture enterprises in most developing countries. Therefore, there is an urgent need to search for other suitable alternatives from

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nonconventional plant protein sources for the replacement of SBM in fish diet.

Jatropha curcas (L.), a member of the family Euphorbiaceae, is a drought-resistant, hardy plant that thrives well on degraded land. *Jatropha* seeds have been extensively investigated as a potential source of oil (400–500 g/kg) for biodiesel production (Singh et al. 2008). The International *Jatropha* Organization has projected that by 2017 there will be around 32.72 million hectares of land cultivated worldwide with *J. curcas*, producing 160 million tons of seeds and 95% of its total production will be concentrated in Asia (Siang 2009; Kumar et al. 2010). The Government of India has initiated a *Jatropha* cultivation project, covering about 400,000 km^2 in line with its vision to meet 20% of its biodiesel requirement (Biswas et al. 2010). Similar initiatives have been taken by other countries like China, Mozambique, and Thailand (Siang 2009). This indicates a potentially huge feedstock supply in the future for the *Jatropha*-based biodiesel industry. Hence, appropriate utilization of the de-oiled *Jatropha* byproduct is vital for the viability and sustainability of the *Jatropha*-based biofuel industry (Saha and Ghosh 2013). *J. curcas* kernel meal (JKM) obtained after oil extraction contains about 58–62% crude protein and has good potential to substitute for SBM in the diets of fish and livestock animals (Makkar and Becker 2008; Kumar et al. 2010). However, despite its protein content, its incorporation in animal diets is restricted because of the presence of highly toxic components – phorbol esters (PEs) and antinutritional factors such as phytate, trypsin inhibitor, and tannin (Makkar and Becker 1998). This toxic component (PEs) cannot be detoxified or inactivated by heat treatment even at higher temperatures (Makkar et al. 1997). However, various detoxification approaches, such as methanol-alkaline extraction (Makkar and Becker 2009), treatment with organic solvent (Devappa et al. 2010), and solid-state fermentation using *Bacillus cereus* (Saha and Ghosh 2013) and *Aspergillus niger* (Shamna et al. 2015), have been used with variable success. Therefore, this study was designed to examine the effects of dietary replacement of SBM with fermented *Jatropha* kernel meal (FJKM) on the hemato-biochemical and histopathological parameters in *Labeo rohita* fingerlings.

Materials and Methods

Preparation of FJKM

Jatropha seeds, procured from Genius Herbs, Coimbatore, India, were deshelled manually to obtain kernels. The kernels were ground with the help of a kitchen grinder and defatted using diethyl ether in a soxhlet extraction apparatus (SOCS PLUS, SCS-08-As, Pelican Equipments, Chennai, India). The defatted meal was detoxified by the solid-state fermentation method, as suggested by Belewu and Sam (2010), using *Aspergillus niger* fungus (MTCC 281, procured from IMTECH, Chandigarh, India) as the fermenting agent. Briefly, the fungus was subcultured in Czapek Yeast Extract Agar, and the fungal spores were harvested using Tween-80 solution and adjusted to 10^7–10^8 spores/mL by serial dilution. Fermentation was conducted for a period of 3 wk on dried JKM at 30 C aerobically. The fermented product was then autoclaved to stop the fungal growth further, cooled, and vacuum dried to obtain FJKM. Autoclaving also inactivates the heat-labile antinutritional factors such as trypsin inhibitor and lectin.

Experimental Diets

Four isonitrogenous (336 g/kg) and isoenergetic (481 Kcal 100/g) diets containing 0, 10, 20, and 30% FJKM in replacement of 0, 33.3, 66.7, and 100% SBM on an equal protein basis were prepared (Table 1). The experimental diets designated as control, T1, T2, and T3 were formulated and prepared using locally available feed ingredients, as shown in Table 2. As per formula, ground ingredients were mixed and dough was prepared, followed by cooking in a pressure cooker for 30 min. Vitamins and mineral mixtures and butylated hydroxyl toluene were added to the dough after cooling so as to prevent their loss in high temperatures. The dough was then subjected to preparation of pellets (2 mm diameter) using hand pelletizer. Finally, the pellets were air dried, followed by oven drying at 60 C until the moisture level reached was less than

TABLE 1. *Proximate composition of JKM, FJKM, and SBM.*

Parameter (g/kg)	JKM	FJKM	SBM
Dry matter	921.0	929.5	965.0
Crude protein	578.5	580.0	505.0
Ether extract	6.2	7.3	32.1
Total ash	96.5	98.4	72.3
Crude fibre	91.2	85.0	59.0
Nitrogen-free extract	227.6	229.4	331.6
Phorbol ester (mg/g)	1.3	N.D.	–
Phytate (% phytic acid equivalent)	8.6	N.D.	2.8
Trypsin inhibitor (TIU mg/g)	21.3	N.D.	N.D.
Tannin (%)	0.38	0.13	0.72

FJKM = fermented *Jatropha* kernel meal; JKM = untreated *Jatropha* kernel meal; N.D. = not detected; SBM = soybean meal.

12%. The dried feeds were packed in airtight polythene zipper bags and stored at 4 C until use.

Acclimation of Fish and Experimental Setup

Fingerlings of Indian major carp, *L. rohita*, were procured from Arrey Fish Farm, Goregaon, Mumbai, India. The fish were transported in a big circular container (500 L) with sufficient aeration to the wet laboratory, ICAR-Central Institute of Fisheries Education, Mumbai, India. Fish were acclimatized to experimental conditions for 15 d and fed with the control diet. The experiment was carried out over a period of 60 d. A total of 180 acclimatized fish (average weight 6.05 ± 0.04 g) were randomly distributed into 12 experimental tubs of 300 L capacity at the rate of 15 fish per tub, following a complete randomized design. Under each treatment group, three replicates were maintained. Aeration was provided round the clock throughout the experimental period. Daily feeding of fish was carried out to satiation level at 1000 and 1800 h using the respective experimental diets. Uneaten feed, together with feces, was carefully siphoned out manually, without any leaching during siphoning, as described by Kumar et al. (2006). Water quality was monitored throughout the experiment and water-quality parameters were within the optimal range (temperature 23.2–27.7 C, pH 7.3–8.3, dissolved oxygen 6.4–7.6 mg/L, total hardness 223–251 mg/L, NH_3-N 0.07–0.15 mg/L, NO_2-N 0.001–0.006 mg/L, and NO_3-N 0.01–0.05 mg/L).

Sample Collection

The methods for sample collection were similar to those previously described in another study conducted in our laboratory (Jha et al. 2007). At the end of the 60-d feeding trial, blood samples were collected from three randomly selected 12-h fasted fish from each replicate. The fish were anesthetized with clove oil (50 µL of clove oil per liter of water) and blood was drawn from the caudal vein using a tuberculin syringe, which was previously rinsed with 2.7% Ethylenediaminetetraacetic acid (EDTA) solution. Collected blood was then transferred immediately to a test tube containing a thin layer of EDTA powder (as an anticoagulant) and shaken gently in order to prevent hemolysis of blood. For serum, blood was drawn from the caudal vein into a dried Eppendorf tube without using anticoagulant and allowed to stand in a tilted position at room temperature for an hour. The sera were collected after centrifuging at 3000 *g* for 5 min into another Eppendorf tube and kept in –20 C until use.

Hematological Parameters and Blood Glucose Level

Hemoglobin content was determined following the cyanmethemoglobin method (Van Kampen and Zijlstra 1961) using Drabkin solution (Qualigens Diagnostics, division of GlaxoSmithKline Pharmaceutical Ltd, Mumbai, India). Hematocrit was determined on the basis of sedimentation of blood. Heparinized blood (50 µL) was taken in the hematocrit capillary (Na-heparinized) and centrifuged (12,000 *g*, 10 min) in the Hematocrit 210 Hettich Centrifuge (Tuttlingen, Germany) to obtain the hematocrit value. Total erythrocyte count was determined using a hemocytometer (Hendrick 1952) and total leucocyte count was determined following the method of Shaw (1930). Blood glucose level was estimated by the method of Nelson and Somogyi (1945) modified by Oser (Nelson and Somogyi 1945).

TABLE 2. *Formulation and proximate composition of the experimental diets (g/kg).*

Ingredients	Control	T1	T2	T3
SBM	300	200	100	0
FJKM	0	100	200	300
FM	50	50	50	50
GNOC	260	240	220	200
Maize flour	100	100	120	140
Wheat bran	119.8	139.8	139.8	139.8
Rice flour	80	80	80	80
Sunflower oil	30	30	30	30
Cod liver oil	30	30	30	30
CMC	10	10	10	10
BHT	0.2	0.2	0.2	0.2
Vitamins and minerals mix[1]	20	20	20	20
Proximate composition				
Moisture (g/kg)	64.0 ± 0.4	66.3 ± 1.2	63.8 ± 0.3	64.2 ± 1.7
CP	338.1 ± 1.7	337.2 ± 2.1	339.0 ± 2.7	332.5 ± 5.3
EE	87.5 ± 5.3	93.0 ± 3.4	97.3 ± 3.1	95.1 ± 5.1
TA	71.6 ± 4.2	75.5 ± 3.7	77.0 ± 3.5	79.5 ± 4.0
CF	72.0 ± 1.5	76.3 ± 2.1	79.4 ± 2.6	75.1 ± 1.3
NFE	430.8 ± 7.2	418.0 ± 3.2	407.3 ± 4.1	417.8 ± 4.5
GE (Kcal 100/g)[2]	479.94 ± 0.9	481.13 ± 0.6	483.08 ± 0.6	479.89 ± 1.2

BHT = butylated hydroxyl toluene; CF = crude fibre (g/kg); CMC = carboxymethyl cellulose; CP = crude protein (g/kg); EE = ether extract (g/kg); FJKM = fermented *Jatropha* kernel meal; FM = fishmeal; GNOC = ground nut oil cake; NFE = nitrogen-free extract (g/kg); SBM = soybean meal; TA = total ash (g/kg).

[1]Composition of vitamins and mineral mix (PREEMIX PLUS, Himedia, Mumbai, India) (quantity/2.5 kg): vitamin A, 55,00,000 IU; vitamin D3, 11,00,000 IU; vitamin B2, 2000 mg; vitamin E, 750 mg; vitamin K, 1000 mg; vitamin B6, 1000 mg; vitamin B12, 6 mcg; calcium pantothenate, 2500 mg; nicotinamide, 10 g; choline chloride, 150 g; Mn, 27,000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 mg; L-lysine, 10 g; DL-methionine, 10 g; selenium, 125 mg; vitamin C, 2500 mg.

[2]Calculated gross energy (GE, Kcal 100/g): (5.64 × CP [g 100/g] + 9.44 × EE [g 100/g] + 4.11 × TC [g 100/g]).

Serum Protein, Nitroblue Tetrazolium Assay, and Histopathology

Serum samples were analyzed for total protein by the biuret method (Reinhold 1953), and albumin was determined by the bromocresol green binding method (Doumas et al. 1971) using diagnostic kits (total protein and albumin kit, Innoline™ Merck Specialities Pvt, Ltd, Mumbai, India). Globulin was calculated by subtracting albumin values from total serum protein, and albumin value was divided by the globulin value to obtain the albumin/globulin ratio. The nitroblue tetrazolium assay was used to quantify the respiratory burst activity of the phagocytes, following the method of Secombes (1990) as modified by Stasiak and Baumann (1996) using an Enzyme Linked Immunosorbent Assay (ELISA) reader. Histopathological examination of the liver and intestine was carried out following the method described by Kumar et al. (2011). In summary, histological samples were fixed in Bouin's fluid for 48 h. After dehydration and embedding in paraffin, serial sections of 5 µm thickness were prepared and processed for conventional histopathological studies. Hematoxylin and eosin stain was used for staining the tissue sections.

Statistical Analysis

All data were subjected to one-way ANOVA using SPSS version 16, and Duncan's multiple range test was used to determine the significant differences between the means. Comparisons were made at the 5% probability level.

Results and Discussion

The results of the effect of dietary inclusion of FJKM as a substitute for SBM protein on hematological parameters are presented in Table 3. A significantly lower ($P < 0.05$) hematocrit value

TABLE 3. *Hematological parameters and blood glucose level of* Labeo rohita *fed different experimental diets.*[1,2]

Treatment	Hb (g/dL)	RBC (10^6 cells/mm^3)	WBC (10^3 cells/mm^3)	Hct (%)	Glucose (g 100/mL)
Control	5.35 ± 0.03	1.45 ± 0.04	468 ± 0.04	17.15^{c} ± 0.13	44.17 ± 0.61
T1	5.29 ± 0.07	1.32 ± 0.08	477 ± 0.04	19.25^{b} ± 0.28	42.93 ± 0.56
T2	5.27 ± 0.09	1.47 ± 0.11	430 ± 0.03	20.72^{a} ± 0.05	42.66 ± 0.38
T3	5.26 ± 0.07	1.39 ± 0.06	470 ± 0.05	20.43^{a} ± 0.07	42.84 ± 0.54

Hb = hemoglobin; RBC = red blood cells; WBC = white blood cells; Hct = hematocrit.
[1]Mean values in the same column with different superscripts differ significantly ($P < 0.05$).
[2]Data expressed as mean ± SE, $n = 6$.

was observed in the control group compared with the higher value recorded in the FJKM groups. Although the observed increase in hematocrit values in *L. rohita* in response to dietary FJKM remains unclear, hematocrit is often used as an indicator of health in fish (Kumar et al. 2010). No significant differences ($P > 0.05$) in the values of hemoglobin, red blood cell, and white blood cell counts, and blood glucose were observed. These findings closely agree with other studies in which processed JKM or SBM were evaluated as protein sources in the diet of fish. For instance, the hematological parameters of common carp, *Cyprinus carpio*, fed diets in which 50 or 75% of FM protein in the control diet was replaced by either detoxified JKM (DJKM) or SBM were unaffected after 8 wk of feeding trial (Kumar et al. 2010). Likewise, with the exception of a significant reduction in blood glucose levels, no differences in such parameters were found in rainbow trout, *Oncorhynchus mykiss*, fed diets in which DJKM replaced 50 or 62.5% of FM protein in the control diet (Kumar et al. 2011).

The lack of an effect in the serum glucose level observed in this study could be attributed to the fact that dietary inclusion of FJKM up to a 30% level in replacement of SBM did not show any adverse effect in *L. rohita* fingerlings. Similar observations were reported by Akinleye et al. (2012) when SBM and heat-treated JKM were fed to Nile tilapia, and by Glencross et al. (2004) when yellow lupin meal was fed to Atlantic salmon and rainbow trout.

Total protein content in the blood is often considered as a good indicator of health and nutritional status in fish (Martinez 1976). Serum protein contains important protein of nutritive function (albumin) and plays a significant role in the immune response (globulin) of an animal (Kumar et al. 2010). In comparison with the control, fish of T1, T2, and T3 did not show any significant difference in serum total protein and albumin contents and globulin levels (Table 4). This indicates a similar nutritional and immunological status among the experimental groups (Congleton and Wagner 2006). This result is consistent with Kumar et al. (2010), who observed no significant effect on the immune response of *C. carpio* fed 50 and 75% DJKM. Further, the respiratory burst activities showed a similar trend.

The histological section of the intestine showed normal histo-architecture, well-defined

TABLE 4. *Biochemical profile of* Labeo rohita *fed different experimental diets.*[1,2]

Treatment	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	A/G ratio	NBT
Control	3.79 ± 0.22	0.78 ± 0.05	3.01 ± 0.17	0.26^{b} ± 0.01	0.43 ± 0.01
T1	3.57 ± 0.11	0.74 ± 0.01	2.83 ± 0.11	0.26^{b} ± 0.01	0.42 ± 0.01
T2	3.57 ± 0.07	0.88 ± 0.04	2.68 ± 0.06	0.33^{a} ± 0.02	0.43 ± 0.01
T3	3.55 ± 0.07	0.77 ± 0.03	2.78 ± 0.10	0.27^{ab} ± 0.02	0.43 ± 0.01

A/G = albumin/globulin; NBT = nitroblue tetrazolium.
[1]Mean values in the same column with different superscripts differ significantly ($P < 0.05$).
[2]Data expressed as mean ± SE, $n = 6$.

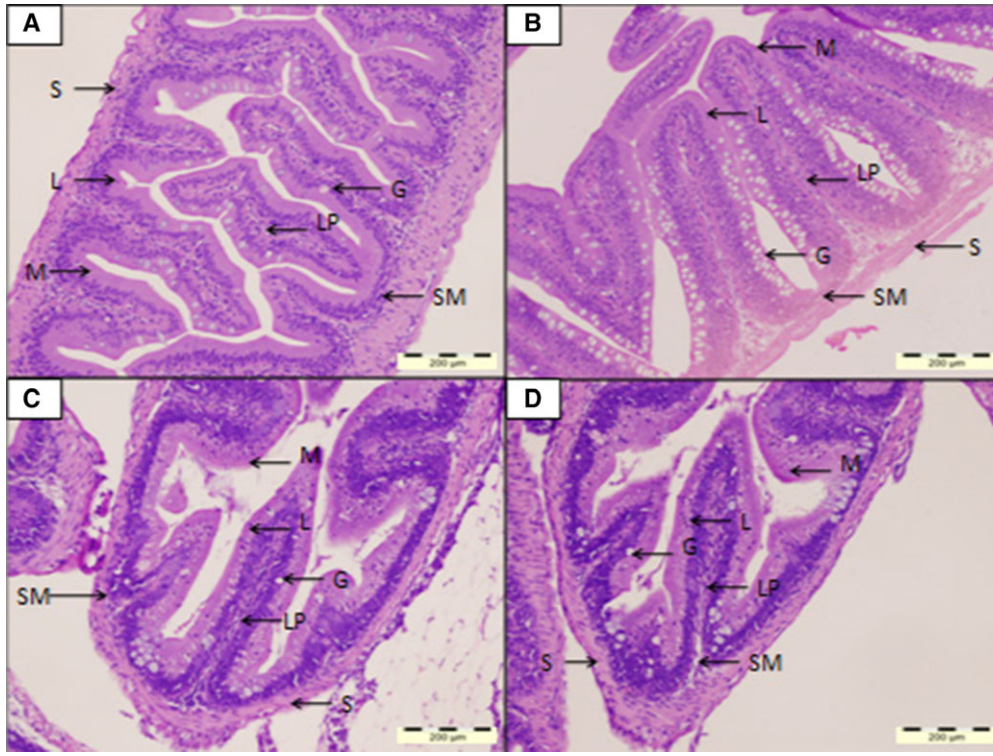


FIGURE 1. *Intestine ((A) control, (B) T1 group, (C) T2 group, and (D) T3 group) of* Labeo rohita *showing normal histo-architecture, well-defined intestinal villi and serosa (S), submucosa (SM), mucosa (M), lamina propria (LP), goblet cells (G), and lymphocyte (L). Staining procedure: hematoxylin and eosin stain. [Color figure can be viewed at wileyonlinelibrary.com].*

intestinal villi, and distinct goblet cells in the fish from the control group (Fig. 1A). An almost similar histological pattern was observed in the intestinal sections of fish from the FJKM groups (Fig. 1B–D). The liver section of the fish from the control group showed normal histo-architecture with centrally placed, round basophilic nuclei and eosinophilic cytoplasm (Fig. 2A). The liver sections of fish from FJKM groups revealed no significant alteration in histology as compared with liver histology of fish from the control group (Fig. 2B–D). As the control and treatment group fish showed no major histo-architectural alterations in intestine and liver histology, it can be stated that the dietary inclusion of FJKM did not negatively affect the intestinal tissues of fish during the experimental feeding. Our histological observation is in accordance with the report of Kumar et al. (2010), in which the authors did not find any distinct changes in the liver morphological integrity in *C. carpio* fed with DJKM-based diet. The histo-architecture of the hepatocytes and hemato-biochemical parameters recorded in this study indicated that JKM subjected to microbial fermentation was adequately detoxified. In addition, considering that no harmful effects were noticed in the FJKM treatment groups, our findings indicate that replacing SBM with FJKM exert no adverse effects to fingerlings of *L. rohita*.

Conclusion

We conclude that up to 30% FJKM can be incorporated in diets of *L. rohita* fingerlings without any detrimental effect on fish health, indicating its potential as an alternative protein source for aquafeed. Further evaluations of this plant-based ingredient in advanced juvenile or adult stages of *L. rohita* are warranted.

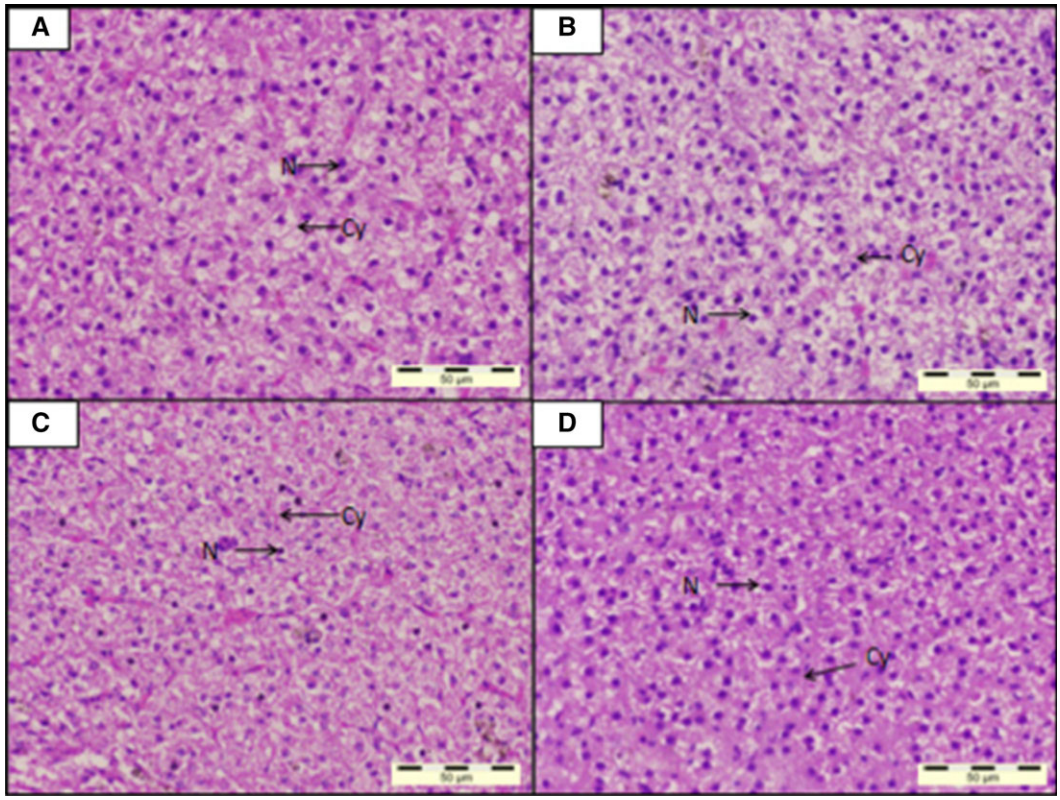


FIGURE 2. *Liver ((A) control, (B) T1 group, (C) T2 group, and (D) T3 group) of* Labeo rohita *showing apparently normal histo-architecture with centrally placed round basophilic nuclei (N) and eosinophilic cytoplasm of the hepatocytes (Cy). Staining procedure: hematoxylin and eosin stain. [Color figure can be viewed at wileyonlinelibrary.com].*

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